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Identification of F2RL3 Gene Methylation Induced by Cigarette Smoking in Acute Myocardial Infarction

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Abstract : Acute Myocardial Infarction (AMI) has complex pathophysiology and influenced by multiple factors. Several recent studies at an epigenetic level conclude that AMI has a strong correlation with changes in methylation patterns of genes associated with IMA, one of which is the F2RL3 gene. This gene encoding for PAR-4, of the receptor for thrombin and widely involved in the several mechanisms of platelet activation, coagulation and has a strong correlation with atherosclerosis. The aim of this study is to investigate the correlation of methylation level on F2RL3 gene caused by cigarette smoking on AMI events. Sixteen AMI patients were collected from Saiful Anwar Hospital, Malang, Indonesia. The whole genome was extracted from patient's blood, then converted with bisulfite conversion method. CpG island in cg03636183 from F2RL3 gene was amplified with a pair of specific primers to obtain specific amplicon containing CpG island. This amplicon then performed with direct sequencing and analyzed by Sequence Scanner v1.0 and BioEdit software. Our result shows that eight AMI patients (50%) having a smoker while eight others (50%) nonsmoker. Three CpG sites can be analyzed which is CpG site 2, CpG site 3, and CpG site 4. There are no differences between methylation level and pattern of F2RL3 gene on AMI patients that induced by cigarette smoking which each group has same methylation levels up to 91,67% respectively. DNA methylation is a complex epigenetic mechanism which is influenced by various factors, not cigarette smoking alone.
Keywords: Acute Myocardial Infarction, cigarette smoking, methylation level of F2RL3 gene.

Introduction

Cardiovascular disease is still the main causes of death in the population of the worldwide, including in Indonesia. Data from the World Health Organization (WHO) in 2012 showed that 31% of death cases from the global deaths in the world were caused by cardiovascular disease[1]. The Acute Myocardial Infarction (AMI) is one type of cardiovascular disease that has the highest prevalence compared with another type of cardiovascular disease. Research at the Harapan Kita Hospital, Indonesia in 2008 showed that there were 7 patients per day attacked by AMI and 120 people per year are under 40 years old.Data from Saiful Anwar Hospital Malang, Indonesia indicates that the IMA is the second leading cause of death with a mortality rate of 16.6% per year.

The term myocardial infarction reflects on cell death of cardiac myocytes caused by ischemia, which is the result of a perfusion imbalance between supply and demand[2, 3]. This disease has a variety of risk factors such as age, gender, heredity, diabetes mellitus, hypertension, obesity, lack of physical activities, stress and

cigarette smoking that believed to increase the prevalence of IMA[4,5]. From these various risk factors, cigarette smoking is believed to have a strong correlation in initiating occurrence of IMA with several pathways.

Cigarette smoke contains the number of gaseous and particulates phase that can initiate atherosclerosis and thrombosis condition by increasing oxidative stress, inflammation, endothelial dysfunction, increasing platelet aggregation, prothrombotic conditions, modification lipid profiles and altering DNA methylation related diseases [6,7,8]. Previous Genome-wide DNA methylation studies concluded that cigarette smoking leads extensive genome-wide changes in DNA methylation and altering methylation of genes related to coronary artery disease[8,9]. Results of these studies reinforced the hypothesis that cigarette smoke is one of the strong environmental factors in initiating changes in DNA methylation.

F2RL3 [*coagulation factor II (thrombin) receptor-like 3*] is a gene located on chromosome 9 and has two exons (NCBI, NC 000019.10). These gene encoding for Protease-Activated Receptor 4 (PAR-4), is a thrombin receptor known to be involved in the several pathways of the coagulation system, initiating platelet aggregation as well as the target of antiplatelet agents[10,11,12]. Methylation level of F2RL3 gene believed to be a contributing factor in one of the pathway in initiating of the IMA occurrence. Previous epigenetic studies concluded that hypomethylation on F2RL3 has a strong correlation with cancer and cardiovascular disease[13,14] and use as one of a predictor for mortality[15].

Until now there has been no study that investigated the gene methylation level F2RL3 caused by smoking that triggering the occurrence of AMI in Indonesian. So this study was designed to investigate the methylation level and pattern of F2RL3 gene induced by cigarette smoking in acute myocardial infarction.

Materials and Methods

Subject Study

Sixteen AMI patients were collected from Saiful Anwar Hospital, Malang, Indonesia during July to December 2015. Subjects were taken based on inclusion criteria including male, current or past diagnosis of dyslipidemia, has levels of LDL cholesterol over 160 mg/dl, levels of triglycerides over 150 mg/dl, levels of HDL cholesterol over 40 mg/dl, smokers or non-smokers, experiencing typical chest pain (angina pectoris) and confirmed by electrocardiogram (ECG).

DNA Methylaton Analysis

CpG island in cg03636183 of F2RL3 gene was amplified with a pair of specific primers: 5'-aggaagagagGGTTTATTAGTAGTATGGTGGAGGG-3' (sense) and 5-cagtaatacgactca ctatagggagaaggctACTTCTAAACTAAATACCCACCAAA-3' (antisense) (Zhang et al, 2014). Twenty five mL total reaction consisted of 22,5 mL Invitrogen PCR master mix; 1,5 ng of DNA template and 0,5 µl of of sense and antisense primer respectively.

Reaction volume then amplified with PCR Thermal Cycler for 35 cycles with five main stages including pre-denaturation at 94 °C for 2 minutes, denaturation at 94 °C for 15 seconds, annealing at 57^{o}C for 45 seconds, extension at 72 C 1 for 1 minute, and final extension at 72 °C for 10 minutes. PCR products then analyzed qualitatively with 1.5% agarose gel then performed with direct sequencing to confirm and analyze the methylation level of F2RL3 gene at cg03636183.

DNA was isolated from patients blood performed with Geneaid gSYNC™ DNA Extraction Kit (www.geneaid.com). Briefly, DNA isolation consisting of five main step including sample preparation by adding 300 mL of whole blood with RBC Lysis Buffer, cell lysis by adding GB Buffer, DNA binding by adding absolute ethanol, washing by adding W1 Buffer and Wash Buffer, and elution by adding elution buffer. The whole genome then analyzed qualitatively by 1 % agarose gel.

The whole genome then converts with bisulfite solution performed by EZ DNA methylation-Gold™ Kit (www.zymoresearch.com). The aim of this step is to convert unmethylated cytosine to thymine. Briefly, there are five major steps: first DNA preparation by adding DNA template with CT Conversion Reagent. The second denaturation by denaturing the DNA template at 98^{o}C for 10 minutes, 53 ^{o}C for 30 minutes, 53 ^{o}C for 6 minutes, 37 °C for 30 minutes then incubating at 4 °C for 20 hours. Third bisulfite deamination by adding M-

Binding Buffer and M-Desulphonation Buffer. Fourth washing by adding M-Wash Buffer and fifth elution by adding M-elution buffer to collect bisulfite-treated DNA.

Data Analysis

Amplicon containing CpG island of F2RL3 gene then analyzed using Sequence Scanner v1.0 and Bioedit Allignment Sequence Editor to determine to total CpG sites were methylated from total samples were used. Methylation level is determined by calculating the total CpG sites were methylated in F2RL3 genes at cg03636183. Methylation level then compared among IMA smokers and IMA non-smokes to determine the effect of cigarette smoking on methylation levels and pattern of F2RL3 gene in acute myocardial infarction.

Result

Baseline Characteristics of IMA Subjects

During June to December 2015, sixteen male subjects with AMI were collected from the Cardiovascular Care Unit (CVCU) of Saiful Anwar hospital, Malang, Indonesia. The data in Table 1 shows the baseline characteristics of the subjects. Based on this data can be described that AMI subjects who involved in this study have mean of age 54.07 ± 6.33 years, mean of Body Mass Index (BMI) 23.25 ± 3.36 kg/m^2, mean of total cholesterol 199.69 ± 49.04 mg/dl, mean of HDL levels 37.38 ± 12.13 mg/dl, mean of LDL levels 132.06 ± 51.46 mg/dl and mean of triglyceride levels 116.5 ± 62.64 mg/dl.

Table 1. Baseline Characteristics of IMA Subjects

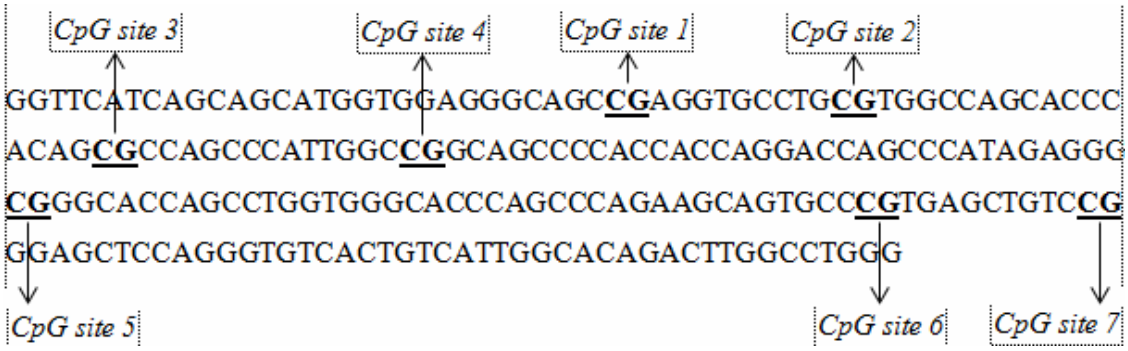
Variable	Value
Age (years)	54.07 ± 6.33
BMI (kg/m^2)	23.25 ± 3.36
Total Cholesterol (mg/dl)	199.69 ± 49.04
HDL (mg/dl)	37.38 ± 12.13
LDL (mg/dl)	132.06 ± 51.46
Triglycerides (mg/dl)	116.5 ± 62.64
Hypertension [n (%)]	5 (31.25)
Dyslipidemia [n (%)]	11 (68.75)
Family history [n (%)]	2 (12.5)
Smoker [n (%)]	8 (50%)

Several other risk factors in the occurrence of IMA were also considered in this study, including hypertension, dyslipidemia, family history and smoking history. 31,25 % of study subjects had hypertension; 68.75 % of study subjects experienced with dyslipidemia; 12,5% of study subjects had a family history of AMI and 50% of the study subjects had a history as a smoker.

Methylation Level of F2RL3 Gene

All of the subjects then grouped into two which is AMI smokers and AMI non-smokers. From sixteen subjects, eight subjects are a smoker, while eight others are a non-smoker. The aim of these grouping is to determine the effect of cigarette smoking on methylation patterns and levels of F2RL3 gene and this correlation with AMI occurrence.

Amplification process using specific primers at cg03636183 of F2RL3 gene produced 206 base pairs of amplicons. This CpG island contains 7 CpG site as shown in Figure 1.



(Zhang et al, 2014)

Figure 1. CpG island of F2RL3 gene at cg g03636183

The result of this study showed that there are no differences in methylation levels and patterns of F2RL3 gene on cg03636183 among AMI smokers and AMI non-smokers. At CpG site 2, there are 6 subjects (75%) are in a methylated state both in AMI smokers and AMI non-smokers while 2 subjects (25%) are in a unmethylated state both in AMI smokers and AMI non-smokers. At CpG site 3 and 4, there are 8 subjects (100%) are in a methylated state both in AMI smokers and AMI non-smokers. The comparison of methylation patterns of F2RL3 gene at cg03636183 between AMI smokers and non-smokers shown in Table 2.

Table 2. Comparison of methylation patterns and levels of F2RL3 gene at cg03636183 between AMI smokers and non-smokers

Status	n	CpG 2		CpG 3		CpG 4	
		Methylated [(n)%]	Un-methylated [(n)%]	Methylated [(n)%]	Un-methylated [(n)%]	Methylated [(n)%]	Un-methylated [(n)%]
Smoker	8	6 (75)	2 (25)	8 (100)	0	8 (100)	0
Non smoker	8	6 (75)	2 (25)	8 (100)	0	8 (100)	0

Discussion

Baseline characteristics of the subjects

Acute myocardial infarction is one type of cardiovascular disease which is characterized by various symptoms. This disease can be triggered by multiple factors. Several predisposing factors that generally have a strong correlation with the AMI incidence including of age, sex, BMI, total cholesterol, the level of HDL cholesterol, the level of LDL cholesterol,the level of triglyceride, dyslipidemia, family history and cigarette smoking.

Sixteen subjects were involved in this study consisted of the male have an average of age 54.07 ± 6.33 year. Previous studies have shown that males had higher prevalence rates of IMA than females in the same range of age. However, the prevalence of women affected by IMA will be increase after experiencing by menopause due to declining of estrogen regulatory control on the cardiovascular system[16,17,18].

Subjects in this study had an average of BMI 23.25 ± 3.36 kg/m^2 and it is classified as the normal category. The proportion of the population are classified as an obese is havingBMI more than 30 kg/m^2, while BMI between 25-29.9 kg/m^{2}is classified as an overweight. Increasing of BMI will be correlated with the increasing of blood pressure, blood cholesterol, and the diabetes risk[19,20,21]. Data from The International Collaborative Study on Hypertension in Blacks (ICSHIB) shows the correlation between BMI and blood pressure. The groups that have BMI less than 24 kg/m^2 had a percentage of hypertension incidence by 15% and rising to 35% in increasing of BMI average by 39 %.

The average of lipid profile in this study is 199.69 ± 49.04 mg/dl for total cholesterol, 12.13 ± 37.38 mg/dl for HDL, 132.06 ± 51.46 mg/dl for LDL, and 116.5 ± 62.64 mg/dl for triglycerides. Symptoms of IMA typically indicated by the modification of lipid profile including increased total cholesterol, LDL, triglycerides also decreased of HDL which leading to dyslipidemia. HDL is known to act as a cardioprotective agent by binding and transporting of cholesterol inside the artery walls, reducing the excess oxidation, reducing the vascular inflammation and thrombosis, improving endothelial cell function, promoting the improvement of endothelial cells, increasing insulin sensitivity and returning the cholesterol to the liver[22,23,24]. The low level of HDL is an early indication of the decline of its role as a cardioprotective agent.

Eight subjects (50%) had a history as a smoker. Several recent studies have shown that cigarette smoke has a strong correlation to initiate cardiovascular disease trough several pathways. Not to be understood with certainty about the pathophysiology of cigarette smoke in initiating of IMA incidence, but it is known that a variety of particulate matter in cigarette smoke can cause lipid modification, vasomotor dysfunction, inflammatory endothelial cells, insulin resistance, increased ROS, thrombus formation, and DNA methylation modification[6,7,25].

DNA Methylation

DNA methylation is one of the epigenetic mechanisms characterized by their covalent bond of a methyl group (CH3) with cytosine bases in the promoter region and forming a CpG island[26,27]. DNA methylation plays an important role in the embryonic development, protection of the genome integrity and regulation of gene expression[28,29]. Therefore when this epigenetic information is not properly established or maintained will cause the growing number of diseases such as cancer, tumor, and cardiovascular diseases[30,31,32,33]. So the study on DNA methylation is essential to understanding the pathophysiology and the link of the diseases thoroughly and deeply.

The results of our study showed that there are no differences in methylation pattern and level of an F2RL3 gene in IMA patients between smokers and non-smokers.DNA methylation is a complex epigenetic mechanism that is not influenced by one factor alone. Several recent studies proved that DNA methylation can be influenced by environmental factors such as diet, air quality, drugs and exposure to environmental chemicals[34,35,36,37] with various mechanisms. In this study, these factors can not be controlled and it is thought to contribute in changing of methylation pattern and level of F2RL3 gene.

Cigarette smoke contains a number of toxic chemicals that believed can influence the DNA methylation. Several previous studies have shown that cigarette smoke has a correlation with alteration of DNA methylation in by causing hypomethylation conditions that lead to development or progression of various diseases[38,39,40].

The results of these studies ensuring that DNA methylation is a complex mechanism that is affected by a number of factors.These results also indicate that the cigarette smoke alone is not a dominant factor in initiating the DNA methylation in the IMA. There are other factors that work individually or together in initiating occurrence of IMA. Our suggestion is necessary to conduct further research by measuring the level of expression of PAR-4 in the serum of AMI patients.

Conclusion

There are no differences in methylation patterns and levels of F2RL3 gene at cg03636183 caused by smoking in AMI patients treated in Saiful Anwar hospital, Malang. DNA methylation is a complex epigenetic mechanism which is not only influenced by cigarette smoking but there are other various factors.

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